Costs of resistance to fungal pathogens in genetically modified wheat

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Abstract: Aims: Many resistance genes against fungal pathogens show costs of resistance. Genetically modified (GM) plants that differ in only one or a few resistance genes from control plants present ideal systems for measuring these costs in the absence of pathogens. Methods: To assess the ecological relevance of costs of pathogen resistance, we grew individual plants of four transgenic spring wheat lines in a field trial with three pathogen levels and varied the genetic diversity of the crop. Important Findings: We found that two lines with a Pm3b transgene were more resistant to powdery mildew than their sister lines of the variety Bobwhite, whereas lines with chitinase (A9) or chitinase and glucanase (A13) transgenes were not more resistant than their mother variety Frisal. Nevertheless, in the absence of the pathogen, both the GM lines of Bobwhite as well as those of Frisal performed significantly worse than their controls, i.e. Pm3b1 and Pm3b2 had 39% or 53% and A9 and A13 had 14% or 23% lower yields. In the presence of the pathogen, all GM lines except Pm3b2 could increase their yields and other fitness-related traits, reaching the performance levels of the control lines. Line Pm3b2 seemed to have lost its phenotypic plasticity and had low performance in all environments. This may have been caused by very high transgene expression. No synergistic effects of mixing different GM lines with each other were detected. This might have been due to high transgene expression or the similarity between the lines regarding their resistance genes. We conclude that costs of resistance can be high for transgenic plants with constitutive transgene expression and that this can occur even in cases where the non-transgenic control lines are already relatively resistant, such as in our variety Frisal. Transgenic plants could only compete with conventional varieties in environments with high pathogen pressure. Furthermore, the large variability among the GM lines, which may be due to unpredictable transgene expression, suggests that case-by-case assessments are necessary to evaluate costs of resistance.

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Running title

Costs of resistance

Abstract

Aims

Many resistance genes against fungal pathogens show costs of resistance. Genetically modified (GM) plants that differ in only one or a few resistance genes from control plants present ideal systems for measuring these costs in the absence of pathogens.

Methods

To assess the ecological relevance of costs of pathogen resistance, we grew individual plants of four transgenic spring wheat lines in a field trial with three pathogen levels and varied the genetic diversity of the crop.

Important Findings

We found that two lines with a *Pm3b* transgene were more resistant to powdery mildew than their sister lines of the variety Bobwhite, whereas lines with *chitinase* (A9) or *chitinase* and *glucanase* (A13) transgenes were not more resistant than their mother variety Frisal. Nevertheless, in the absence of the pathogen, both the GM lines of Bobwhite as well as those of Frisal performed significantly worse than their controls, i.e. *Pm3b*#1 and *Pm3b*#2 had 39% or 53% and *A9* and *A13* had 14% or 23% lower yields. In the presence of the pathogen, all GM lines except *Pm3b*#2 could increase their yields and other fitness-related traits, reaching the performance levels of the control lines. Line *Pm3b*#2 seemed to have lost its phenotypic plasticity and had low performance in all environments. This may have been caused by very high transgene expression. No synergistic effects of mixing different GM lines with each other were detected. This might have been due to high transgene expression or the similarity between the lines regarding their resistance genes.

We conclude that costs of resistance can be high for transgenic plants with constitutive transgene expression and that this can occur even in cases where the non-transgenic control lines are already relatively resistant, such as in our variety Frisal. Transgenic plants could only compete with conventional varieties in environments with high pathogen pressure. Furthermore, the large variability among the GM lines, which may be due to unpredictable transgene expression, suggests that case-by-case assessments are necessary to evaluate costs of resistance.

Key words: Powdery mildew, resistance genes, transgene expression, transgene stacking, yield

Introduction

Plants interact with their environment in various ways. They have to compete with their neighbours and endure abiotic stresses and pathogen attacks. Natural selection can improve competitiveness and stress resistance. However, there are no wild plants with resistances against all possible pathogens (Bergelson and Purrington 1996a), an observation consistent with the idea of a trade-off between performance and defence (Herms and Mattson 1992). Genes that increase resistance against pathogens may be costly for a plant in the absence of pathogens. A meta-analysis showed that resistant plants had lower fitness than non-resistant ones in approximately half of 88 studies considered (Bergelson and Purrington 1996a). It is important to understand the mechanism leading to such costs and how these affect plant–pathogen systems, as such knowledge is relevant for basic ecology as well as for agricultural ecosystems (Brown 2002).

Fitness costs that are associated with pathogen resistance are difficult to measure. Resistance genes are often linked to other genes, making it difficult to elucidate single-gene costs of resistance. This problem can be avoided by using transgenic (genetically modified = GM) plants that differ only in one or a few known genes from their original genetic background (Burdon and Thrall 2003; Purrington 2000). Thus, transgenic crop plants may serve as model systems for ecologists interested in costs of pathogen resistance, even though they may differ in some aspects from wild plants.

Few studies to date have measured costs of resistance in transgenic plants (Burdon and Thrall 2003; Bergelson *et al.* 1996b, Purrington, 2000; Romeis *et al.* 2007; Tian *et al.* 2003, Vila-Aiub *et al.* 2009). Resistance costs of transgenes have been found

79 in some but not all of these studies (Snow *et al.* 1999). Even if such costs exist, they
80 have to be put into the right context. There are very few studies (e.g, Brunner *et al.*
81 2011) that varied the pathogen pressure, which is necessary to study the ecological
82 relevance of costs associated with resistance genes. The pathogen level can itself be
83 influenced by the plant community, which can either facilitate or slow down the spread
84 of epidemics. In particular, genetic diversity for pathogen resistance in a plant stand can
85 reduce the pathogen pressure and therefore increase the performance at the level of the
86 population and of individual plants (Mundt 2002; Schmid 1994; Wolfe 2000; Zeller *et*
87 *al.*, *in review*). However, we did not find any published reports where the influence of
88 pathogen pressure and community diversity on plant performance and costs of
89 resistance were evaluated in combination.
90 We therefore performed a field trial with four transgenic and two non-transgenic
91 lines of spring wheat *Triticum aestivum* L. that belonged either to the variety Bobwhite
92 or Frisal. The GM Bobwhite lines *Pm3b*#1 and *Pm3b*#2 harboured a *Pm3b* transgene
93 against powdery mildew *Blumeria graminis* f.sp. *tritici* (DC.) Speer, whereas the Frisal
94 lines A9 and A13 had either a *chitinase* or a *chitinase* and a *glucanase* gene,
95 respectively, to induce quantitative fungal resistance. These transgenic lines were
96 produced from commercially available Bobwhite or Frisal plants, which we took as
97 controls. We established three fungal infection treatments. One third of the studied
98 plants were sprayed with fungicide to prevent powdery mildew infection, to allow
99 measurement of potential costs of resistance in the absence of the pathogen.
100 Furthermore, plants were naturally or artificially infected with powdery mildew to
101 obtain different pathogen infection levels. We worked with individual plants that were
102 hand-seeded into plots containing either Bobwhite or Frisal lines of varying genetic

diversity (0, 1 or 2 GM lines). The factorial design, combining the different wheat lines with fungal infection and genetic diversity treatments, allowed us to address the following questions: (i) are there differences between GM and non-GM lines and between different GM lines? (ii) are there costs of resistance in the absence of pathogens? (iii) does the mixing of plant lines and therefore the increase of genetic diversity increase resistance and performance and are there interactions between fungal infection and diversity treatments?

Materials and methods

Genetically modified wheat

We used six spring wheat lines of the Mexican variety Bobwhite SH 98 26 (Brunner *et al.* 2011; Lindfeld *et al.* 2011; Peter *et al.* 2010; von Burg *et al.* 2010; von Burg *et al.* 2011; Zeller *et al.* 2010) and the Swiss variety Frisal (Bieri *et al.* 2003, Kalinina *et al.* 2011) for our experiment. Two GM and one non-GM line were chosen from each variety.

The GM lines of Bobwhite harboured a *Pm3b* transgene in different position on the genome, each derived from different transformation events. *Pm3b* confers race-specific resistance to powdery mildew and was obtained from the hexaploid wheat variety Chul (Yahiaoui *et al.* 2004). The lines, which were named *Pm3b#1* and *Pm3b#2*, were generated by biolistic transformation (Pellegrineschi *et al.* 2002). The *Pm3b* gene was cloned under the control of the *Zea mays* L. (maize) ubiquitin promoter (Christensen and Quail 1996). More detailed information can be found in previous studies (Zeller *et al.* 2010, Brunner *et al.* 2011). Presence of the transgenes was confirmed by Southern hybridization analysis (Southern 2006). The GM lines contained

127 the *Pmi* gene as well as one complete copy of *Pm3b*, which segregated as a single
128 Mendelian locus in the T1 generation. Two *Pm3b* lines were multiplied to T5 and used
129 for the field experiment. The level of transgene expression was assessed by quantitative
130 real time PCR using RNA isolated from leaves of field-grown plants. It revealed that
131 *Pm3b* genes in the lines *Pm3b*#1 and *Pm3b*#2 were expressed constitutively and that the
132 mean expression level was 11 and 55 times higher than in the variety Chul, where this
133 gene is expressed naturally (Brunner *et al.* 2011; Zeller *et al.* 2010).
134 The two transgenic lines with the genetic background of the variety Frisal
135 contained genes from barley which are known for their anti-fungal effect and the
136 constitutive or inducible expression of pathogenesis-related genes (Zhu *et al.* 1994).
137 Line A9 harboured a *chitinase* and A13 both a *chitinase* and a β-1,3-*glucanase*
138 transgene (Bliffeld *et al.* 1999). Both lines were generated by biolistic transformation. A
139 maize ubiquitin promoter (Christensen and Quail 1996) was used for the *chitinase* and
140 an actin promoter from rice (McElroy *et al.* 1990) for the β-1,3-*glucanase*. The
141 expression of the transgenes *chitinase* and β-1,3-*glucanase* was analyzed by SDS-
142 PAGE and Western blotting of intercellular wash fluid from mature leaves, and in later
143 generations on total protein from seedling leaves (Bieri *et al.* 2003). Both lines were
144 multiplied to T6 in the glasshouse in order to verify stable expression of the transgenes.
145
146 *Field experiment*
147 The field experiment took place at an agricultural research station in Zurich-
148 Reckenholz, Switzerland, at 440 m above sea level. It started in March 2009 and lasted
149 until beginning of August 2009. Three powdery-mildew treatment blocks, each with
150 twelve 1.0 x 1.3 m plots, were sown with seeds of the six lines described above

(Supplementary Figure S1). Besides the monocultures, six plots with 50:50 mixtures consisting of *Pm3b*#1/Bobwhite control, *Pm3b*#2/Bobwhite control, *Pm3b*#1/*Pm3b*#2 as well as A9/Frisal control, A13/Frisal control, A9/A13 were sown to assess mixture effects. In each plot five rows with a distance of 20 cm between them were sown at a density of 400 seeds per m^2 using a Seedmatic system (Hege 90, Hege Maschinen, Eging am See, Germany). To assess the performance of individual plants it was essential to know the line identity of plants in mixture plots. We therefore inserted short sections consisting of 7 seeds ("seed islands") of known identity by hand into the prepared rows. This was done right after the machine sowing. Each island was shifted slightly relative to the machine-sown row to allow the removal of machine-sown seedlings immediately after emergence (see Supplementary Figure S1). Monocultures received one and mixture plots two islands. This planting procedure guaranteed that the hand-sown seeds in these seed islands had an almost identical competitive environment as the machine-sown seeds. Three out of the seven planted seedlings per island (position 2, 4, 6) were marked with a label after emergence.

The three fungal infection treatments were fungicide application and natural and artificial mildew infection. Fungicide plots were sprayed three times with the fungicide Prosper (500g l^{-1} Spiroxamine; Leu + Gygax AG, Birmenstorf, Switzerland). This allowed keeping the plots almost completely free of powdery mildew. In the natural infection plots, neither artificial inoculation nor fungicides were applied. All untreated plots were infected strongly by powdery mildew during the field experiment. The plots with artificial powdery mildew infection were bordered with "spreader rows" of the susceptible conventional winter wheat variety Kanzler. The plants of the spreader rows had been pre-grown and inoculated with powdery mildew isolate 96224 in the

175 glasshouse. The distance between spreader rows and plots was 80 cm. The powdery
176 mildew isolate 96224 had been collected between Winterthur and Kloten (Switzerland)
177 in 1996 (Brunner *et al.* 2010; Srichumpa *et al.* 2005) and was known to be avirulent on
178 *Pm3b* (Yahiaoui *et al.* 2009). A second batch of inoculated plantlets were produced and
179 planted one month later. The three fungal infection treatments were separated from each
180 other by a 4-m wide border crop of spring triticale to reduce cross-contamination.
181 Based on a nutrient assessment, different amounts of nitrogen fertilizer were
182 applied before sowing. This resulted in equal nitrogen concentrations (7.5g N m^{-2}) in
183 each block. At the phenological stages 22–29 (Zadoks *et al.* 1974) additional nitrogen
184 was added (3 g N m^{-2} as "Ammonsalpeter 27.5", Lonza, Visp, Switzerland). The natural
185 field soil provided the plants with sufficient phosphorous, potassium and magnesium
186 (81, 176 and 248 mg kg^{-1}). All plots were sprayed with the herbicide cocktail Concert
187 SX (40% Thifensulfurone, 4% Metusulfurone-methyl; Stähler Suisse AG, Zofingen,
188 Switzerland) and Starane super (120 g l^{-1} Bromoxynil, 120 g l^{-1} Ioxynil, 100 g l^{-1}
189 Fluroxypyr-metilheptil-ester; Omya Agro AG, Safenwil, Switzerland) at the beginning
190 of May. Insecticide Karate Zeon (100g l^{-1} Lambda-Cyhalothrin; Syngenta Agro AG,
191 Dielsdorf, Switzerland) against the wheat stem fly (*Chlorops pumilionis* Bjerk.) was
192 applied at the beginning of May and repeated 2 weeks later.
193
194 *Response variables*
195 The degree of powdery mildew infection (Eyal *et al.* 1987) was assessed 32, 45, 59 and
196 80 days after germination. Based on these data, we calculated the "Area Under Disease
197 Progress Curve", AUDPC (Jeger and Viljanen-Rollinson 2001). AUDPC is the amount
198 of disease integrated over the time period of interests. It is based on the trapezoidal rule

for calculating areas (Jeger and Viljanen-Rollinson 2001). After ripening, all marked plants were cut at ground level and separated into vegetative and reproductive parts (spikes). Vegetative and reproductive parts were then dried at 80 and 25 C°, respectively, and weighed. We then threshed the reproductive parts and obtained the seed mass which is equivalent to seed yield. The seeds obtained from all spikes of a plant were counted by hand. Vegetative mass was calculated by subtracting the seed mass from the total biomass. Furthermore, plant height was measured at the highest point of the plant from the soil, 80 days after germination.

Data analysis

We analysed the data with mixed-model analysis of variance using the classical ANOVA as well as the REML (Restricted Maximum Likelihood) method with the statistical software GenStat (VSN International Ldt). Results were almost identical and thus only the REML analyses are presented in this paper because they are considered to yield better results when missing values occur in a data set (Payne et al. 2010). In contrast to the classical method, which fits a mean for each level of a random-effects term, the REML method directly estimates the variance components of such terms. We used blocks, the block x fungal treatment interaction, plots nested within this interaction and islands nested within plots as random-effects terms in the analysis. Using these random-effects terms and the REML approach ensured that fixed-effects terms were automatically tested against appropriate error terms (Payne et al. 2010). Terms for fixed effects were fitted with hierarchical and factorial models as follows.

First, we used an “all hierarchical” treatment/line model that sequentially, i) divided the line effects (six levels) into a contrast between Bobwhite and Frisal plants,

ii) added within each variety the fungal infection treatment (three levels) as a iia) contrast between fungicide and mildew infection and a iib) contrast between natural and artificial infection within the latter, iii) added the two remaining line-effects contrasts iiia) control vs. GM lines and iiib) differences between the two GM lines within each variety (Model 1; Figure 1, Table S1). Second, we used a "factorial sub-model" after the initial contrast i) between Bobwhite and Frisal for each of the two varieties separately. The sub-model contained the main effects of fungal infection treatment, divided into the two contrasts iia) and iib), the main effects of the two remaining line-effects contrasts iiia) and iiib) and the corresponding four contrast interactions (Model 2; Table S2). The advantage of these contrast formations was that they yielded focused single-degree of freedom tests (Rosenthal and Rosnow 1985). As recommended by these authors, we used this approach of focused comparison instead of post-hoc multiple comparison tests.

Two additional terms were added to these two models to assess the influence on the target plants of the number of GM-lines (GM-richness 0, 1 and 2) or the proportion of GM-plants (GM-concentration 0, 50, 100%) per plot. Since these two contrasts were partly confounded with each other, their fitting sequence was alternated in two separate runs of the analyses. Furthermore, these contrasts were either fitted before or after the effects of the lines and the fungal infection treatment. Fitting GM-richness and -concentration first in the models allowed an assessment of their influence "ignoring" confounding effects of the lines (effects of fungal infection treatment were not confounded with GM-richness or GM-concentration and therefore in this case the fitting sequence did not matter). Fitting GM-richness and -concentration after the fungal infection treatment and line effects allowed an assessment of their influence

247 "eliminating" confounding effects of the lines (see e.g. McCullagh and Nelder 1989 for
248 the ignoring/eliminating terminology).
249 To understand better the effects of fungal infection treatments and GM-richness
250 and GM-concentration within each, Bobwhite or Frisal, we repeated all analyses with
251 datasets restricted to either of the two varieties. However, we mostly present results
252 from the full model.
253 Residual plots were examined to check if the assumptions of normality and
254 homoscedasticity were fulfilled. Seed yield, vegetative mass and seed number were
255 square-root transformed and x^2 transformation was necessary for plant height. Back-
256 transformed means and standard errors from the REML output were used to draw the
257 figures. The critical significance level was 0.05 in all analyses.
258 Since several of the measured traits correlated with each other, we also
259 performed a Multivariate Linear Mixed Model (MLMM) to test for the overall
260 significance of fungal infection treatment and line effects. The five traits AUDPC, plant
261 height, seed yield, vegetative mass and seed number were combined in a single analysis.
262 Transformed data were used for the MLMM analysis.
263
264 **Results**
265 *Powdery mildew infection*
266 The spring wheat variety Bobwhite was more susceptible to powdery mildew than the
267 old Swiss variety Frisal ("Bobwhite vs. Frisal": $P<0.001$; Figure 2a and Supplementary
268 Table S1). The repeated spraying with fungicide reduced mildew infections by a factor
269 of 6.2 for Bobwhite and by a factor of 5.4 for Frisal plants ("Fungicide vs. Mildew"
270 within Bobwhite or within Frisal both $P<0.001$, see Supplementary Table S1). The

271 natural and artificial mildew treatment levels did not differ significantly from each other
272 with regard to mildew infection, both within Bobwhite or within Frisal. Nevertheless,
273 we assume that the composition of the pathogen community differed between these two
274 treatment levels, because the artificial infection was done with one particular powdery
275 mildew strain. The Bobwhite GM lines *Pm3b*#1 and *Pm3b*#2 were less susceptible to
276 powdery mildew than the non-transgenic Bobwhite control line in all three fungal
277 infection treatments (83, 52 and 61% less mildew in fungicide-treated, natural infection
278 and artificial infection plots, respectively). *Pm3b*#2 had 36% less powdery mildew than
279 *Pm3b*#1 in the plots with natural infection ($P<0.001$; "*Pm3b*#1/2 in Natural" in
280 Supplementary Table S1). There was no such difference between the two Bobwhite GM
281 lines in the plots with artificial infection where a mildew strain avirulent for *Pm3b*
282 genes was released.

283 Mildew infections decreased with increasing GM-concentration in the plots
284 (GM-concentration fitted before line effects: $P<0.001$, data not shown). Results for GM-
285 richness were less clear. GM-rich plots had significantly less mildew when GM-
286 richness was fitted before GM-concentration. However, this signal was lost when GM-
287 concentration was fitted before GM-richness. To understand why GM-concentration and
288 GM-richness reduced the mildew infection levels in diverse plots, we performed further
289 analyses. We fitted GM-concentration and GM-richness after fungal infection treatment
290 and line effects and interactions and therefore eliminated these (see Material and
291 Methods). As a result, the significant results from above disappeared (see
292 Supplementary Table S1), which means that the decreased powdery mildew infection
293 can be explained by the different pathogen resistance levels of the individual lines (line
294 effects). The GM-Frisal lines A9 and A13 showed no increased pathogen resistance

295 when compared to plants of the Frisal control line and also no differences for GM-
296 concentration or GM-richness. The mixing of lines *Pm3b*#1 with *Pm3b*#2 or A9 with
297 A13 did therefore not lead to synergistic reduction of powdery mildew infection levels.
298
299 *Fungal infection treatment effects and differences between GM and control lines in*
300 *these (all hierarchical model)*
301 Plants of the variety Bobwhite differed from Frisal in all traits (MLMM, "Bobwhite vs.
302 Frisal": $P<0.001$). The performance of Bobwhite and Frisal plants depended strongly on
303 the fungicide or mildew treatment levels and therefore on the pathogen pressure
304 (MLMM, "Fungicide/Mildew" for Bobwhite and Frisal both with $P<0.001$). Neither
305 Bobwhite nor Frisal lines performed differently in plots with natural as compared with
306 artificial infection. We describe the Bobwhite results first, followed by Frisal.
307 The fungicide application increased plant height within the Bobwhite variety
308 ($P=0.002$; "Fungicide vs. Mildew in Bobwhite" for plant height in Supplementary Table
309 S1). However, there were no overall positive effects on seed yield or vegetative mass
310 because of line-specific responses to the fungicide application. Seed yields of plants of
311 the Bobwhite control line and the GM line *Pm3b*#2 were 31% and 13% higher,
312 respectively, under fungicide application, whereas they were 28% lower for plants of
313 the GM line *Pm3b*#1.
314 Bobwhite GM lines reacted differently to fungicide spraying compared to
315 Bobwhite control lines ($P=0.005$; "Fungicide/Mildew x BW/GM within variety
316 Bobwhite" for seed yield in Supplementary Table S2). When comparing the Bobwhite
317 control line with the mean of the two Bobwhite GM lines in the fungicide-treated plots,
318 we found that the latter had 42% fewer seeds ($P<0.001$), 46% lower seed yield

(P<0.001), 34% lower vegetative mass (P<0.001) and 7% lower plant height (P=0.002; "BW/GM in Fungicide" in Supplementary Table S1). The seed yield of line *Pm3b*#1 was 39% and that of line *Pm3b*#2 was 53% lower than that of the Bobwhite control line. These results indicate that the Bobwhite GM lines, in contrast to the control line, did not benefit from the absence of the pathogens. Bobwhite GM lines had on average less seeds than Bobwhite control in the natural infection treatment level (P=0.016; "BW/GM in Natural" for seed number in Supplementary Table S1).

Frisal lines that were sprayed with fungicide grew taller than unsprayed plants ("Fungicide vs. Mildew within Frisal" for plant height: P=0.003; Supplementary Table S1). As for the Bobwhite lines, the two Frisal GM lines had on average 20% fewer seeds (P=0.026), 18% lower yield (P=0.043) and 6% lower plant height (P<0.001) than the control line ("Frisal/GM in Fungicide"; Supplementary Table S1) in the sprayed plots. We found that the yield of line A9 was 14% and that of line A13 was 23% lower when compared to Frisal control. No such differences were found for plants growing in plots with natural or artificial infection.

Differences between GM-lines (factorial submodel)

Although the two GM lines of Bobwhite, *Pm3b*#1 and *Pm3b*#2, had the same transgene, they had very different phenotypes (MLMM, "*Pm3b*#1/2": P<0.001). *Pm3b*#2 had 19% fewer seeds (P=0.051), 41% lower seed yield (P<0.001), 19% lower vegetative mass (P=0.058) and a 5% reduced height (P<0.001) compared with *Pm3b*#1 ("*Pm3b*#1/2"; Supplementary Table S2). In addition to this overall difference, the two GM lines also showed different responses to the two mildew treatments levels ("Fungicide/Mildew x *Pm3b*#1/2" for vegetative mass: P=0.038; Supplementary Table S2). This was due to a

higher relative performance of *Pm3b*#1 in plots with mildew than with fungicide whereas no such response was found for line *Pm3b*#2. However, even the GM line *Pm3b*#1 never reached the performance of control plants in fungicide plots. The yield of unsprayed *Pm3b*#1 was 21% and that of *Pm3b*#2 59% lower than that of the Bobwhite control line in the fungicide treatment level.

Also in the variety Frisal the two GM lines, A9 and A13, had different phenotypes (MLMM, "A9/A13": $P<0.001$). Plants of line A9 were 4% shorter ($P<0.001$) and had 18% more seeds ($P=.015$) than A13 ("A9/A13"; Supplementary Table S2). As for the Bobwhite GM lines, also the Frisal GM lines could never reach the yields of sprayed Frisal control plants. Unsprayed A9 plants had 20% and unsprayed A13 plants had 27% lower seed yields than sprayed plants of the Frisal control line.

Effects of GM-concentration and GM-richness

The genetic diversity of the plot into which the tested plants were sown influenced their performance. Plants in plots with higher GM-concentration had fewer seeds ($P<0.001$), lower seed yield ($P=.005$) and were shorter ($P<0.001$) than plants in plots with higher GM-concentration. To understand why GM-concentration had mostly negative effects on fitness-related traits, we fitted GM-concentration and GM-richness after line and fungal infection treatment effects and interactions and therefore eliminated these (see Materials and Methods). As a result, all significant results from above disappeared (see Supplementary Tables S1, S2). By looking at the data we could see that the good performance of Bobwhite control and the bad performance of line *Pm3b*#2 underlie most of the concentration and richness effects. No synergistic effects caused by the mixing of lines *Pm3b*#1 with *Pm3b*#2 or A9 with A13 were detected.

Discussion

Powdery mildew infection

Our results show that the two tested spring wheat varieties differed from each other. Bobwhite lines proved to be more susceptible to powdery mildew than the Swiss variety Frisal. This might have to do with different breeding aims and the origin of these varieties. In Switzerland, where powdery mildew is a serious plant disease, breeders have favoured resistant varieties whereas this was not necessary in Mexico where no natural epidemics occur (Lillemo *et al.* 2006). Frisal entered the official variety list of Switzerland in 1987. After the release, the susceptibility to powdery mildew and leaf rust increased during the nineties (M. Winzeler, personal communication). Frisal was subsequently taken off the market in 2006. It is therefore not surprising that not only Bobwhite but also Frisal lines were infected by this pathogen. The GM lines *Pm3b*#1 and *Pm3b*#2 proved to be more resistant to powdery mildew than their genetic background Bobwhite. No such differences were detected in the A9 and A13 lines which were produced from Frisal. This may be because Frisal control lines were already relatively resistant to powdery mildew. It is conceivable that this native resistance could not be improved by additional resistance genes. This result, however, contrasts with laboratory results where A9 was less susceptible to powdery mildew than Frisal (Bieri *et al.* 2003). Hence, these results demonstrate the importance of field trials.

Since we worked in a natural environment it was not possible to remove the omnipresent natural mildew spores. However, the fungicide used in the fungicide treatment level reduced powdery mildew infections in all plots to almost zero. This allowed us to assess the influence of the pathogen pressure on fitness-related traits and

391 unintended effects. The difference between the natural and artificial treatment levels
392 was less prominent. There was no overall difference in pathogen abundance (AUDPC)
393 between these two treatment levels, although the artificial infection started before the
394 natural infection (data not shown). It is conceivable that climatic conditions and not the
395 start of the inoculation mainly affected the spread and growth of powdery mildew.
396 However, it is likely that the artificially introduced mildew isolate 96224 was more
397 common in artificial than in natural infection plots. This strain is avirulent for (i.e. can
398 not attack) the two Bobwhite GM lines *Pm3b*#1 and *Pm3b*#2. We therefore expected
399 less mildew in these plots than in the naturally infected ones. Indeed, line *Pm3b*#1
400 proved to be more resistant in the artificially than in the naturally inoculated plots. Line
401 *Pm3b*#2, however, was highly resistant in both and this could have been due to the very
402 high transgene expression levels of this line that made it even resistant to a "non-target"
403 powdery mildew strain. Brunner *et al.* (2011) argued that high expression does provide
404 some degree of quantitative resistance against different strains of powdery mildew.
405 Besides the mildew treatment levels, we analysed the influence of plant diversity
406 on individual plants within a plot. Plants in plots with high concentrations of resistant
407 GM lines had less powdery mildew than plants in plots with the susceptible Bobwhite
408 control line. This effect could be explained by the presence or absence of the susceptible
409 Bobwhite line. One reason to include diversity treatments into our experimental design
410 was to assess possible synergistic effects caused by the mixing of different GM lines.
411 There are several publications that show improved pathogen resistance in fields with
412 mixed varieties (Finckh *et al.* 2000; Mundt 2002; Wolfe 2000). However, we found no
413 indications that mixed *Pm3b*#1 and *Pm3b*#2 plots were more resistant against powdery
414 mildew than monocultures of these GM lines with identical transgenes but different

415 expression levels. There are at least two explanations for this. Either the influence of the
416 mixed background was not strong enough to affect the plants which themselves
417 belonged to uniform seed islands or these lines were too similar to allow synergistic or
418 complementary effects. The same might be true for the Frisal lines. Although not
419 genetically identical, all three Frisal lines were similarly resistant against powdery
420 mildew in all three fungal infection treatments. Hence, in the absence of variability, no
421 synergistic effects should perhaps have been expected.
422
423 *Costs of Resistance*
424 If a transgene would induce complete pathogen resistance without any costs, we would
425 expect GM lines to perform as well as non-resistant control lines in absence of
426 pathogens. We found, however, that all four GM lines performed worse than their
427 Bobwhite and Frisal control lines in fungicide-treated plots. In fact, none of the lines
428 ever reached the level of the non-GM control lines even in the un-sprayed plots. This
429 indicates that *Pm3b* as well as *chitinase* and *glucanase* transgenes cause costs of
430 resistance. We found that the disadvantage of GM lines, as expected, decreased in plots
431 with high pathogen levels.
432 Whereas costs of resistance might explain why these GM lines did not reach the
433 level of the control lines in the absence of the pathogen, this does not explain why line
434 *Pm3b*#1 performed worse in the fungicide than in the mildew treatment levels. One
435 explanation could be that the chemicals of the fungicide interacted with the transgene or
436 its products. Increased sensitivity to fungicide was described already earlier in a
437 glasshouse study (Zeller *et al.* 2010). The sum of costs of resistance and fungicide
438 sensitivity could have caused the large fitness reductions in lines *Pm3b*#1 and *Pm2b*#2.

Since it is not possible to remove a common pathogen from a field without the use of pesticide, one would have to revert to closed systems without pathogen presence to study costs of resistance separate from potential fungicide effects. However, costs of resistance might not be visible under conditions that are optimal for plant growth. A better approach than closed systems might be to carry out field trials in areas where the targeted pathogen does not occur naturally, or to stress the plants in the closed system.

Whereas line *Pm3b*#1 performed better in the mildew than in the fungicide treatment presumably due to benefits related to its powdery mildew resistance; *Pm3b*#2 performed poorly in all environments. For this line, costs of resistance seemed to be so high that potential benefits of the transgene were offset in all environments. Line *Pm3b*#1 apparently could retain more plasticity than did line *Pm3b*#2. This difference might be explained by the expression level. Line *Pm3b*#2 is known for much higher transgene expression levels than line Pm3b#1 (Brunner et al. 2011; Zeller *et al.* 2010). It is conceivable that costs of resistance increase with higher expression level because of increased metabolic stress. Besides the high expression levels, it would also be possible that not the gene dosage, but location-dependent interactions of the transgene with the native genome caused these negative effects (Bergelson *et al.* 1996b).

Among the GM Frisal lines, A13 grew taller than A19. Seed yield and seed number were lower in line A13 but these differences were not significant. We could therefore not prove that line A13, which harbours two transgenes, performs worse than line A9 with only one. Further experiments are necessary to assess if the number of transgenes within a single plant increases costs of resistance.

GM plants with high costs of resistance may not be particularly useful in agronomy. They have however one advantage: their risk of spreading uncontrollably in

463 fields or even to natural habitats is very low. It is very likely that such plants would be
464 outcompeted in natural habitats where pathogens are known to fluctuate widely.
465 It should be noted, however, that it would be unlikely for such GM lines with
466 inferior performance to reach the stage of commercialisation. Suitable crop lines are
467 usually selected from a pool of several hundred or even thousands of lines. Plants with
468 poor performance in the field, as the one's which we used here, can still be discarded at
469 a late testing stage, i.e. after they have been moved from the controlled environment to
470 the field.
471
472 *Diversity effects*
473 Besides the influence of the fungal infection treatments, we studied how the genetic
474 diversity of stands influenced individual plants within these. There are examples from
475 agronomy where increased diversity leads to reduced pathogen susceptibility and
476 transgressive overyielding (Finckh *et al*. 2000; Mundt 2002; Wolfe 2000). If crop
477 varieties or wild plant species are mixed with each other, it is difficult if not impossible
478 to test if particular resistance genes or other phenotypic traits are responsible for these
479 positive diversity effects. Transgenic plants that differ only in single genes can be useful
480 to understand such mechanisms. Hence, we planted either monocultures or mixtures of
481 one GM with one non-GM line or two different GM lines. We found that several
482 fitness-related traits and plant height were influenced by the concentration of GM plants
483 within each plot. However, almost all of these differences could be explained by the
484 presence of a particular line in the corresponding plots. No further benefits of mixing
485 these GM lines with each other were detected. This result is in line with the powdery
486 mildew results discussed above. Individual plants were not less infected with this

pathogen than expected from the monoculture means. The amount of powdery mildew infection seemed to influence the overall performance of our study plants. Thus, because powdery mildew was not reduced more in plots with two GM lines than in plots with only one, we would also not expect positive effects on other traits. Furthermore, high costs of resistance might have concealed such effects. As described in the section above, the GM lines might have been too similar to complement each other, or the lack of mixing in the planted islands could have concealed the effects. Indeed, we found strong diversity effects in a sister study in which we mixed GM lines with different *Pm3* alleles (Zeller *et al.*, *in review*). We recommend, therefore, using more dissimilar transgenic plants for future diversity studies. Furthermore, better mixing might be necessary to obtain good diversity effects.

Conclusions

Our study demonstrates that transgenic plants may differ from their non-GM control lines in many traits and that these differences can be influenced by environmental factors (i). There were differences between the Bobwhite GM lines *Pm3b*#1 and *Pm3b*#2 as well as between the Frisal GM lines A9 and A13. The latter might be explained by differences in the introduced gene construct. The lines *Pm3b*#1 and *Pm3b*#2 share, however, an identical transgene. It is most likely that different expression levels caused by positional effects were responsible for the differences between the two Bobwhite GM lines. In view of all this variation, we conclude that ecological assessments of GM plants should be done on a case-by-case basis (Andow and Zwahlen 2006).

We found that all four tested GM lines suffered from costs of resistance in the absence of the pathogen (ii). Interestingly, even transgenic lines without further increased pathogen resistance compared to already resistant control lines (variety Frisal) showed such negative effects. However, in the presence of the pathogen, three of the four tested GM lines did not differ in their performance from the non-GM control lines. In this case positive effects of the pathogen resistance probably compensated for the negative effects of costs of resistance.

Finally, the diversity of the plant communities influenced pathogen levels and plant performance (iii). However, no synergistic effects were detected. We conclude that the balance between costs and benefits of increased pathogen resistance and therefore the performance of GM plants depends mainly on environmental factors. It is conceivable that transgenic plants with high costs of resistance can outperform conventional lines only in areas with constantly high pathogen pressure. Pathogen populations are known to vary from year to year depending mostly on weather conditions and other factors. Hence, in years of low pathogen pressure, non-resistant plants should have an advantage over resistant plants. One could therefore recommend to cultivate both resistant and non-resistant plants in places with variable pathogen populations.

Supplementary Data

Supplementary Figure S1 shows the experimental design of the field trial. Two Supplementary Tables show summary REML analyses for five different traits. Hierarchical models were used in Supplementary Table S1 and factorial models in

Supplementary Table S2. Supplementary data are available at the *Journal of Plant Ecology* online.

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649
650

Figure 1. Hierarchical line/treatment model used in the analysis. Circles indicate varieties or lines whereas rectangles represent treatments.

Figure 2. Effects of fungicide and natural and artificial powdery mildew infection on performance of GM and non GM-wheat. The left column shows the non-transgenic variety Bobwhite (dashed line, round symbols) and two transgenic lines *Pm3b*#1 (solid lines, square symbols) and *Pm3b#2* (solid lines, triangular symbols). The right column shows the non-transgenic variety Frisal (dashed line, round symbols) and two transgenic lines A9 (solid lines, square symbols) and A13 (solid lines, triangular symbols). A–E present the level of powdery mildew infection, seed number, seed yield, vegetative mass and plant height. Light grey lines were drawn to make transgene x fungal infection treatment interactions visible; error bars represent ± 1 standard error (back-transformed, see Material and Methods) and are sometimes hidden behind the symbols.

Figure 1

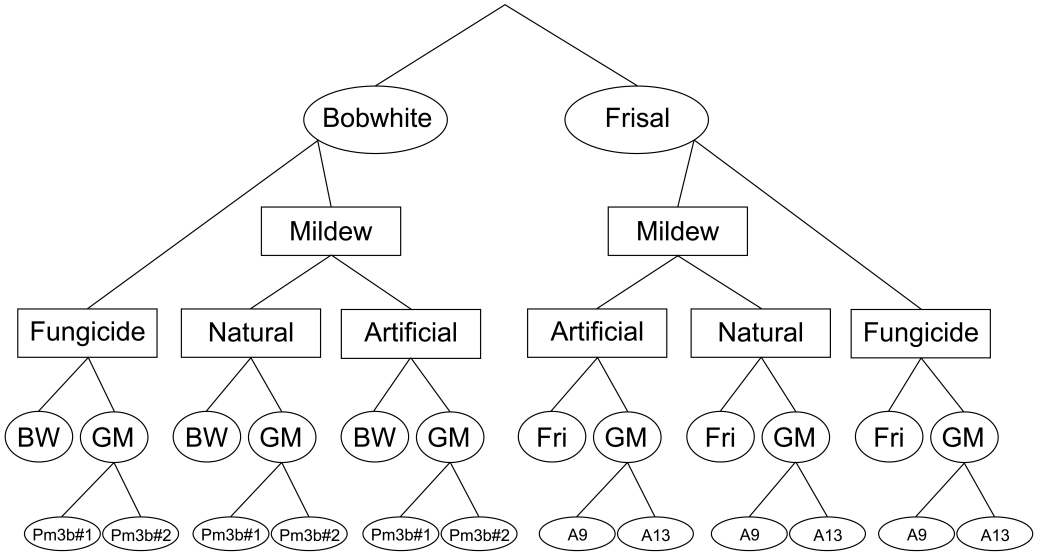

Bobwhite
Frisal
Mildew
Mildew
Fungicide
Natural
Artificial
Artificial
Natural
Fungicide
BW
GM
BW
GM
BW
GM
Fri
GM
Fri
GM
Fri
GM
Pm3b#1
Pm3b#2
Pm3b#1
Pm3b#2
Pm3b#1
Pm3b#2
A9
A13
A9
A13
A9
A13

Figure 2

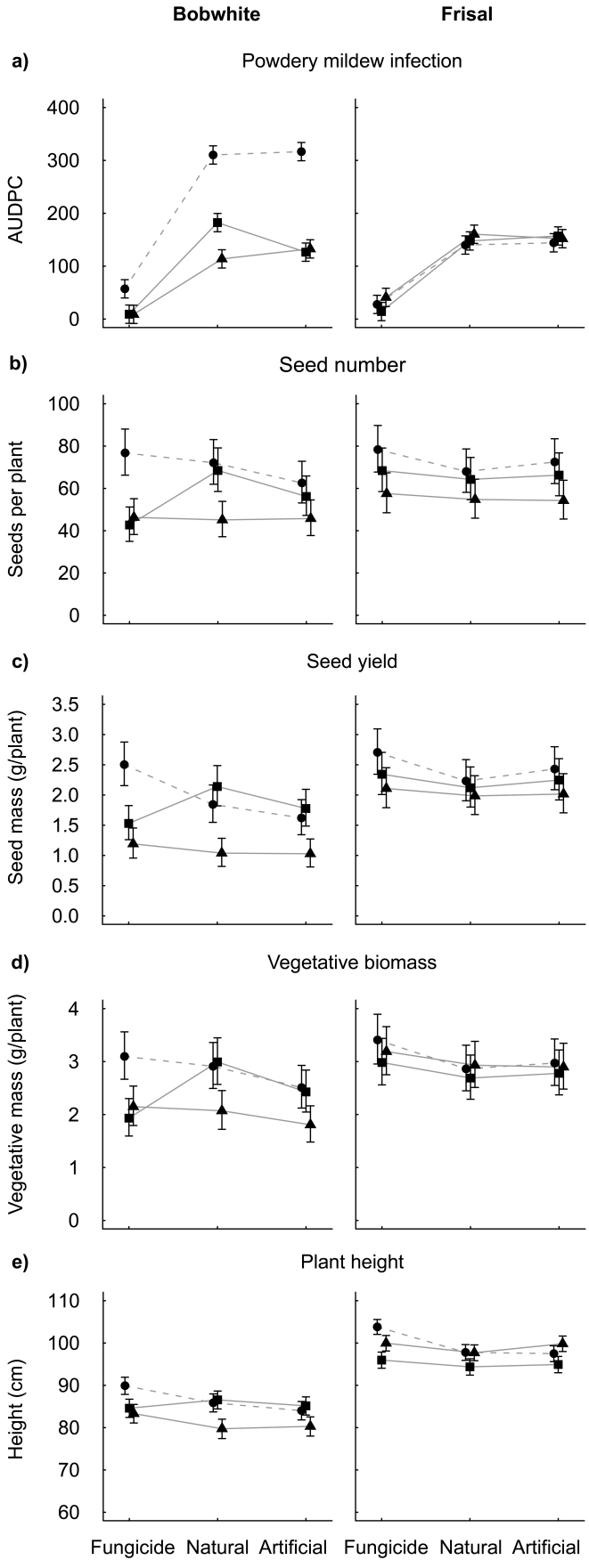

Bobwhite
Frisal
a)
Powdery mildew infection
AUDPC
400
300
200
100
0
b)
Seed number
Seeds per plant
100
80
60
40
20
0
c)
Seed yield
Seed mass (g/plant)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
d)
Vegetative biomass
Vegetative mass (g/plant)
4
3
2
1
0
e)
Plant height
Height (cm)
110
100
90
80
70
60
Fungicide
Natural
Artificial
Fungicide
Natural
Artificial

Table S1. Hierarchical model: summary of REML tables of AUDPC, seed number, seed yield, vegetative mass and plant height. GM richness and GM concentration were alternated. Random terms are not included in the table since their variance components are estimated directly in the REML analyses. The percentage of Wald statistic thus was calculated only for the total of the fixed effects. The total is smaller than 100% because complex interactions were omitted in this table.

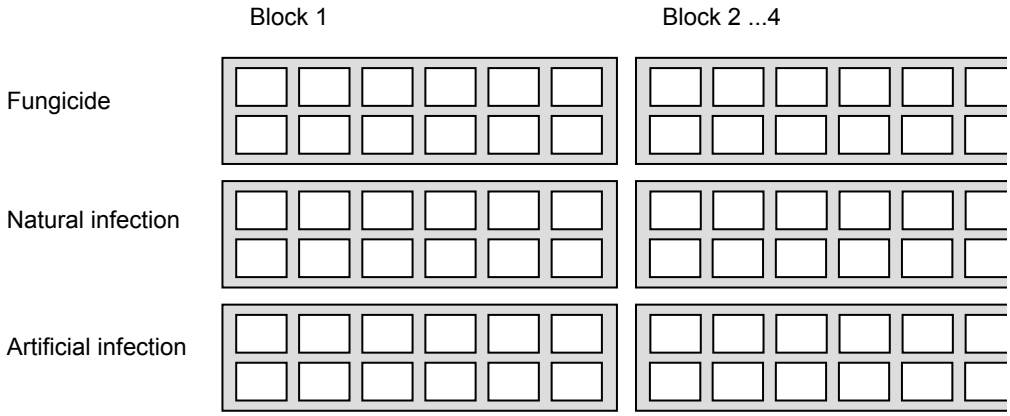
			AUDPC		Seed number		Seed yield		Vegetative mass		Plant height	
	Source of variation	df	Wald %	Chi pr.	Wald %	Chi pr.	Wald %	Chi pr.	Wald %	Chi pr.	Wald %	Chi pr.
	Bobwhite vs. Frisal	1	3.4	<0.001***	8.8	0.026*	32.8	<0.001***	29.8	<0.001***	80.3	<0.001***
Variety Bobwhite (BW)	Fungicide vs. Mildew	1	10.6	<0.001***	1.6	0.349	0.4	0.527	1.1	0.494	0.7	0.095
	BW/GM in Fungicide	1	2.5	<0.001***	32.0	<0.001***	22.2	<0.001***	24.9	<0.001***	2.2	0.002**
	Pm3b#1/2 in Fungicide	1	0.0	0.896	0.3	0.672	3.0	0.082	0.8	0.562	0.1	0.423
	Natural vs. Artifical	1	0.1	0.577	1.8	0.818	1.0	0.316	4.8	0.163	0.0	0.690
	BW/GM in Natural	1	27.8	<0.001***	10.3	0.016*	2.6	0.108	5.7	0.122	0.7	0.086
	Pm3b#1/2 in Natural	1	2.2	<0.001***	9.2	0.022*	16.7	<0.001***	7.4	0.077	1.8	0.005**
	BW/GM in Artificial	1	41.0	<0.001***	3.4	0.165	0.9	0.347	3.3	0.242	0.0	0.767
	Pm3b#1/2 in Artificial	1	0.4	0.118	4.1	0.128	10.4	<0.001***	10.9	0.033*	1.2	0.025*
Variety Frisal	Fungicide vs. Mildew	1	10.5	<0.001***	1.5	0.369	1.3	0.256	4.4	0.187	2.2	0.003**
	Frisal/GM in Fungicide	1	0.0	0.678	8.7	0.026*	4.0	0.043*	3.7	0.216	4.0	<0.001***
	A9/A13 in Fungicide	1	0.4	0.127	3.2	0.176	0.6	0.425	0.8	0.561	1.8	0.005**
	Natural vs. Artifical	1	0.0	0.835	0.2	0.731	0.3	0.573	0.2	0.788	0.1	0.609
	Frisal/GM in Natural	1	0.5	0.091	1.7	0.320	0.2	0.625	0.0	0.992	0.3	0.264
	A9/A13 in Natural	1	0.1	0.490	3.7	0.146	0.5	0.470	0.6	0.624	0.7	0.077
	Frisal/GM in Artificial	1	0.1	0.397	5.4	0.080	1.7	0.183	0.6	0.602	0.0	0.872
	A9/A13 in Artificial	1	0.1	0.435	3.4	0.164	0.5	0.459	0.3	0.722	3.1	<0.001***
Diversity	GM richness	1	0.0	0.835	0.2	0.748	0.2	0.641	0.4	0.687	0.0	0.853
	GM concentration	1	1.6	0.208	0.6	0.557	0.5	0.466	0.3	0.739	0.8	0.073
	GM concentration	1	0.1	0.463	0.1	0.849	0.0	0.849	0.0	0.959	0.3	0.253
	GM richness	1	0.2	0.296	0.7	0.521	0.7	0.399	0.6	0.603	0.5	0.163

Table S2. Factorial model: summary of REML tables of AUDPC, seed number, seed yield, vegetative mass and plant height. GM richness and GM concentration were alternated. Random terms are not included in the table since their variance components are estimated directly in the REML analyses. The percentage of Wald statistic thus was calculated only for the total of the fixed effects. The total is smaller than 100% because complex interactions were omitted in this table.

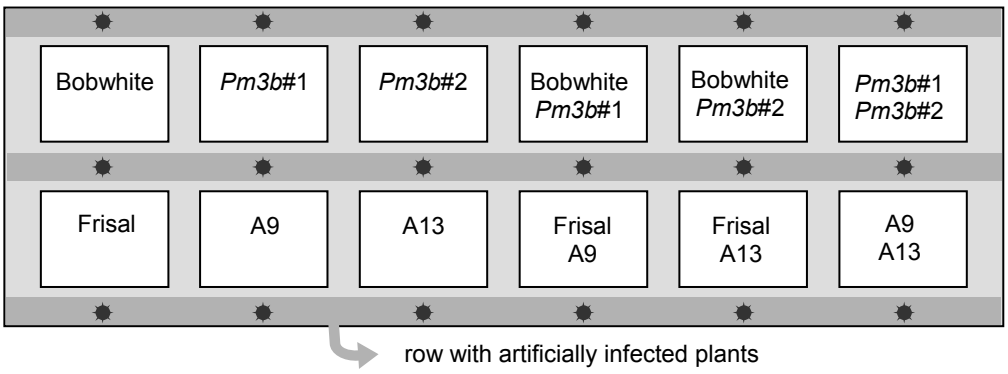
			AUDPC		Seed number		Seed yield		Vegetative mass		Plant height	
	Source of variation	df	Wald %	Chi pr.	Wald %	Chi pr.	Wald %	Chi pr.	Wald %	Chi pr.	Wald %	Chi pr.
	Bobwhite vs. Frisal	1	3.4	<0.001***	8.8	0.026*	32.8	<0.001***	29.8	<0.001***	80.3	<0.001***
Variety Bobwhite (BW)	Fungicide/Mildew	1	10.6	<0.001***	1.6	0.349	0.4	0.527	1.1	0.494	0.7	0.095
	Natural/Artificial infection	1	0.1	0.577	1.8	0.318	1.0	0.316	4.8	0.163	0.0	0.690
	BW/GM	1	58.6	<0.001***	38.2	<0.001***	17.5	<0.001***	28.1	<0.001***	2.0	0.003**
	Pm3b#1/2	1	0.3	0.215	6.7	0.051	27.1	<0.001***	8.6	0.058	2.6	<0.001***
	Fung/Mild x BW/GM	1	12.0	<0.001***	6.5	0.054	7.9	0.005**	5.7	0.121	0.7	0.088
	Fung/Mild x Pm3b#1/2	1	0.1	0.474	6.4	0.056	2.5	0.109	10.2	0.038*	0.5	0.160
	Nat/Art x BW/GM	1	0.6	0.053	0.9	0.465	0.2	0.629	0.2	0.779	0.2	0.314
	Nat/Art x Pm3b#1/2	1	2.3	<0.001***	0.5	0.599	0.3	0.565	0.2	0.770	0.0	0.695
Variety Frisal	Fungicide/Mildew	1	10.5	<0.001***	1.5	0.369	1.3	0.256	4.4	0.187	2.2	0.003**
	Natural/Artificial infection	1	0.0	0.835	0.2	0.731	0.3	0.573	0.2	0.788	0.1	0.609
	Frisal/GM	1	0.5	0.088	14.5	0.002**	4.9	0.026*	2.5	0.307	2.3	0.002**
	A9/A13	1	0.1	0.409	10.3	0.015*	1.7	0.191	1.6	0.410	5.2	<0.001***
	Fung/Mild x Frisal/GM	1	0.1	0.485	0.8	0.492	0.8	0.367	1.5	0.428	1.9	0.004**
	Fung/Mild x A9/A13	1	0.3	0.201	0.0	0.953	0.0	0.958	0.0	0.898	0.0	0.961
	Nat/Art x Frisal/GM	1	0.1	0.549	0.5	0.598	0.3	0.554	0.3	0.717	0.1	0.499
	Nat/Art x A9/A13	1	0.2	0.298	0.0	0.963	0.0	0.992	0.0	0.924	0.4	0.176
Diversity	GM richness	1	0.0	0.835	0.2	0.748	0.2	0.641	0.4	0.687	0.0	0.853
	GM concentration	1	0.3	0.208	0.6	0.557	0.5	0.466	0.3	0.739	0.8	0.073
	GM concentration	1	0.1	0.463	0.1	0.849	0.0	0.849	0.0	0.959	0.3	0.253
	GM richness	1	0.2	0.296	0.7	0.521	0.7	0.399	0.6	0.603	0.4	0.163

Figure S1. Experimental design

A. Block and treatment structure



B. Plots in treatment (example artificial infection)



C. Seed islands in plot (example Bobwhite / *Pm3b*#1 mixture)